EXHIBIT F

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# Identification of a Novel Human Tankyrase through Its Interaction with the Adaptor Protein Grb14*

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**Ruth J. Lyons‡, Róisín Deane‡, Danielle K. Lynch‡, Zheng-Sheng Jeffrey Ye§¶, Georgina M. Sanderson‡, Helen J. Eyre‖, Grant R. Sutherland‖, and Roger J. Daly‡****

*From the ‡Cancer Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, New South Wales 2010, Australia, the §Department of Cell Biology and Genetics, Rockefeller University, New York, New York 10021, the ¶Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, and the ‖Centre for Medical Genetics, Department of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, Adelaide, South Australia 5006, Australia*

**Tankyrase is an ankyrin repeat-containing poly(ADP-ribose) polymerase originally isolated as a binding partner for the telomeric protein TRF1, but recently identified as a mitogen-activated protein kinase substrate implicated in regulation of Golgi vesicle trafficking. In this study, a novel human tankyrase, designated tankyrase 2, was isolated in a yeast two-hybrid screen as a binding partner for the Src homology 2 domain-containing adaptor protein Grb14. Tankyrase 2 is a 130-kDa protein, which lacks the N-terminal histidine/proline/serine-rich region of tankyrase, but contains a corresponding ankyrin repeat region, sterile α motif module, and poly(ADP-ribose) polymerase homology domain. The *TANKYRASE 2* gene localizes to chromosome 10q23.2 and is widely expressed, with mRNA transcripts particularly abundant in skeletal muscle and placenta. Upon subcellular fractionation, both Grb14 and tankyrase 2 associate with the low density microsome fraction, and association of these proteins *in vivo* can be detected by co-immunoprecipitation analysis. Deletion analyses implicate the N-terminal 110 amino acids of Grb14 and ankyrin repeats 10–19 of tankyrase 2 in mediating this interaction. This study supports a role for the tankyrases in cytoplasmic signal transduction pathways and suggests that vesicle trafficking may be involved in the subcellular localization or signaling function of Grb14.**

It is now evident that protein-protein interactions play a critical role in signal transduction, not only mediating recruitment of signaling proteins to receptors and assembly of multiprotein signaling complexes, but also directing the correct subcellular compartmentalization of such complexes and hence providing signal fidelity (1). A variety of protein modules have been identified that mediate these interactions including SH2[1] domains, which bind specific phosphotyrosine-containing peptide sequences; SH3 domains, which target specific proline-rich motifs with a P*XX*P core; and PDZ domains, which interact with the C-terminal consensus (S/T/Y)*X*(V/I) (2, 3). Another module, the PH domain, also mediates intermolecular interactions, but here the targets are predominantly specific polyphosphoinositides and inositol polyphosphates (4). These modules may be found in signaling proteins that possess a catalytic activity (*e.g.* c-Src and phospholipase C-γ); the adaptor class (*e.g.* Grb2), which provide a molecular link to separate effector molecules; and proteins that provide an anchoring or scaffolding function, *e.g.* PSD-95 (1). As well as initiating signaling events, protein-protein interactions are also important in regulating the internalization of cell surface receptors and their subsequent sorting to lysosomal or recycling compartments (5, 6).

The Grb7 family is a group of related SH2 domain-containing adaptors, comprising Grb7, -10, and -14 (7). These proteins share significant sequence homology and a conserved molecular architecture, consisting of a N-terminal region containing the motif P(S/A)IPNPFPEL, a central PH domain-containing region (designated the GM domain), which bears homology to the *Caenorhabditis elegans* protein Mig10 and a C-terminal SH2 domain. The family members differ in their specificity and modes of receptor recruitment. Grb7 binds via its SH2 domain to a variety of receptor tyrosine kinases and tyrosine-phosphorylated proteins, including erbB2, erbB3, and Shc (7–10). In the case of Grb10, most attention has focused on its recruitment by the IR and IGF-1R (7, 11). Grb14 is also bound by the IR (12) and has recently been identified as a fibroblast growth factor receptor 1 target (13). A 50-amino acid region between the PH and SH2 domains (BPS domain) contributes to binding of Grb10 and Grb14 to the IR (11, 12).

The signaling function of the Grb7 family is poorly understood. One role for Grb10 and -14 may be as negative regulators of IR signaling. For example, overexpression of Grb14 reduces insulin-induced DNA and glycogen synthesis (12) and inhibition of insulin-induced insulin-like receptor substrate-1 phosphorylation occurs upon overexpression of hGrb10β[2] (Grb-IR)

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF329696.*

** To whom correspondence should be addressed. Tel.: 61-2-9295-8333; Fax: 61-2-9295-8321; E-mail: r.daly@garvan.org.au.

[1] The abbreviations used are: SH, Src homology; Ab, antibody; AD, activation domain; BD, binding domain; bp, base pair(s); BPS, between pleckstrin homology and Src homology 2; CHX, cycloheximide; DAPI, 4′, 6-diamidine-2-phenylindole; FISH, fluorescence *in situ* hybridization; FTCD, formiminotransferase cyclodeaminase; GM, Grb-Mig; Grb, growth factor receptor-bound; GST, glutathione *S*-transferase; HDM, high density microsome; HPS, histidine/proline/serine-rich; IGF-1(R), insulin-like growth factor 1 (receptor); IR, insulin receptor; IRAP, insulin-responsive aminopeptidase; kb, kilobase(s); LDM, low density microsome; MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PDZ, PSD-95/Dlg/ZO1; PH, pleckstrin homology; SAM, sterile α motif; TRF, telomere repeat binding factor.

[2] The nomenclature used for particular Grb10 isoforms is that proposed by André Nantel following consultation with workers in the field.

(14) or Grb14 (12). However, data supporting a positive role for mGrb10$\alpha$ in insulin-, IGF-1-, and platelet-derived growth factor BB-stimulated mitogenesis have recently been presented (15). It is also likely that the functional role of the Grb7 family extends beyond signal modulation. For example, inhibition of Grb7 expression suppresses the invasive potential of esophageal cancer cells (16), and overexpression of Grb7 and its targeting to focal contacts correlates with increased cell motility (17). Definition of the molecular interactions mediated by Grb7 family proteins, particularly those involving the N-terminal and GM domains, may provide a valuable insight into their signaling mechanism and how it is regulated. With regard to the N-terminal region, the SH3 domain of c-Abl binds the conserved proline-rich motif of Grb10 *in vitro* (18), but an *in vivo* binding partner has yet to be identified.

In this paper we describe the identification of a novel tankyrase, tankyrase 2, as a binding partner for the Grb14 N terminus. Tankyrase was originally identified by virtue of an interaction with the telomeric protein TRF1, and consists of a N-terminal HPS region, 24 consecutive ankyrin-type repeats, a SAM module, and a C-terminal region with homology to the PARP catalytic domain (19). A small fraction of tankyrase co-localizes with TRF1 at telomeres, and tankyrase can ADP-ribosylate TRF1 *in vitro*, leading to a reduction in binding of TRF1 to telomeric DNA. Consequently, one function of tankyrase may be in regulation of telomere function via ADP-ribosylation. However, the majority of tankyrase is extranuclear, and a recent study identified it as a peripheral membrane protein associated with the Golgi, where it localizes to Glut4 vesicles via the IRAP cytosolic domain and acts as a substrate for insulin and growth factor-induced MAP kinase activity (20). Interestingly, tankyrase 2 is also predominantly cytoplasmic and associates with the LDM fraction. The association of tankyrase 2 with Grb14 supports the hypothesis that tankyrases may provide a link between signal transduction pathways and vesicle trafficking.

## EXPERIMENTAL PROCEDURES

*Yeast Two-hybrid Library Screening*—A plasmid construct encoding a Gal4 DNA-BD-Grb14 fusion was generated as follows. The plasmid *GRB14*/pRcCMV$_F$ containing full-length *GRB14* cDNA (21) was digested with *Hin*dIII and Klenow-treated to create blunt ends, and then digested with *Bcl*I to release three fragments of ~1.1, 4.2, and 1.7 kb. The 1.7-kb fragment was isolated and cloned into the *Nde*I (Klenow-treated) and *Bam*HI sites of the yeast expression vector pAS2–1 (CLONTECH, Palo Alto, CA) to generate *GRB14*/pAS2–1 containing an in-frame fusion of full-length Grb14 with the Gal4 DNA-BD. This construct was introduced by electroporation into the yeast strain CG1945 selecting for tryptophan prototrophy. Following preparation of yeast cell extracts by trichloroacetic acid protein extraction, the expression of the fusion protein was verified by Western blot analysis with antibodies directed against the Flag epitope or the Gal4 DNA-BD. The recipient strain was then grown to mid-log phase and a human liver cDNA library in the vector pACT2 (CLONTECH) introduced using the LiAc procedure (22). Transformants were selected for tryptophan, leucine, and histidine prototrophy in the presence of 5 mM 3-aminotriazole and then tested for $\beta$-galactosidase activity by either a liquid culture-based method (Galacto-Light, Tropix, Bedford, MA) or colony lift filter assay (CLONTECH).

Clones scoring positive in the $\beta$-galactosidase assays were subjected to CHX curing to remove the bait plasmid by streaking out on synthetic complete-leu media containing 10 $\mu$g/ml CHX (pAS2–1 contains the *CYH2* gene which restores CHX sensitivity to CG1945 cells). This enabled confirmation of the bait dependence of *LacZ* activation and subsequent isolation of the pACT2 plasmids encoding interacting proteins by standard methodology (23). Back transformations were then performed in which these pACT2 plasmids were introduced into CG1945 strains containing the bait plasmid (*GRB14*/pAS2–1) or constructs encoding non-related Gal4 DNA-BD fusions in order to confirm the specificity of the interactions.

The DNA sequences of the cDNA inserts were then obtained by cycle sequencing (Promega, Annandale, New South Wales, Australia) using pACT2-specific and/or clone-specific primers.

*Analysis of Protein-Protein Interactions Using the Yeast Two-hybrid System*—In order to identify the region of Grb14 that interacts with tankyrase 2, a series of Grb14 deletion mutants were generated by cloning polymerase chain reaction fragments synthesized using the appropriate flanking primers into the vector pAS2–1. These fragments spanned the following regions: N terminus (amino acids 1–110), the central region encompassing the Mig10 homology and the BPS domain (amino acids 110–437), and the N-terminal and central regions (amino acids 1–437). These plasmids were individually transformed into the yeast strain Y190. Following transformation of the resulting yeast strains with the *TANKYRASE 2* cDNA clone L1 in pACT-2, the strength of the interaction was determined by either liquid- or filter-based $\beta$-galactosidase assays. Expression of the constructs was confirmed by Western blotting of yeast extracts with Gal4 DNA-BD- and Gal4 AD-specific antibodies.

In order to investigate the interaction of tankyrase 2 with TRF1, a fragment of tankyrase 2 corresponding to the 10-ankyrin repeat region of tankyrase responsible for TRF1 binding (TR1L12) (19) was expressed as a Gal4 AD fusion in pGAD10 (CLONTECH). Binding of this to LexA fusions of full-length TRF1 and TRF1 lacking the tankyrase binding site (amino acids 1–67) was then performed as described previously (19).

*Library Screening and Clone Characterization*—Following the isolation of the original *TANKYRASE 2* cDNA, further clones were isolated by standard cDNA library screening methodology (24). DNA probes were labeled by random primer extension (Promega) using [$\alpha$-$^{32}$P]dCTP (110 TBq/mmol, Amersham Pharmacia Biotech Pty Ltd, Castle Hill, New South Wales, Australia). Following isolation of phage or phagemid DNA (Promega Wizard kits), sequencing of the cDNA inserts was performed by cycle sequencing. The cDNA cloning strategy was as follows, and further cDNA clone details can be provided upon request. The original *TANKYRASE 2* cDNA isolated from the two hybrid screen (L1) was used as a probe to screen a $\lambda$gt10 human placental cDNA library (5′ Stretch Plus, CLONTECH). This isolated two clones, designated P8 and P12. P8 was ~2.0 kb and provided the C-terminal end of the tankyrase 2 protein sequence. P12 was ~3.5 kb and extended the cDNA sequence 0.9 kb in the 5′ direction. Screening of the human placental cDNA library and a $\lambda$gt11 human small intestine cDNA library (5′ Stretch, CLONTECH) with 5′-located probes led to the isolation of two clones, designated P5 and SI4, respectively, which both extended the sequence further 5′ and provided a putative translation initiation codon. Screening of a $\lambda$ZAP II human fetal brain cDNA library (Stratagene, La Jolla, CA) with a 414-bp probe including the extended sequence isolated two further clones, FB3 and FB11, which confirmed this sequence. Sequence alignments were performed using the program ClustalW.

*Assembly and Transcription/Translation of the TANKYRASE 2 cDNA*—The cDNA was first assembled in the vector Bluescript SK+ (Stratagene) containing alterations to the multiple cloning site (MCS). The MCS was changed by insertion of annealed oligonucleotides 5′-GGCCGCGGATCCCGGCTCGAGCGGGAATTCCATGCCATGGCATGCCAAGCTTTCTAGAG-3′ and 5′-TCGACTCTAGAAAGCTTGGCATGCCATGGCATGGAATTCCCGCTCGAGCCGGGATCCGC-3′ into the *Not*I/*Xho*I sites to provide the modified cloning site *Not*I, *Bam*HI, *Xho*I, *Eco*RI, *Nco*I, *Hin*dIII, *Xba*I and to destroy the original *Xho*I site, creating the vector BSK (ΔMCS). The first 495 bp of *TANKYRASE 2* were obtained as a *Bam*HI/*Xho*I fragment from FB11, and inserted into the *Bam*HI/*Xho*I sites of BSK (ΔMCS) creating BSK(I). The next 840 bp were obtained as a *Xho*I/*Eco*RI fragment from SI4 and cloned into the *Xho*I/*Eco*RI sites of BSK(I) creating BSK(II). The following 1104 bp were obtained as a *Eco*RI/*Nco*I fragment from L1 and inserted into the *Eco*RI/*Nco*I site in BSK(II), creating BSK(III). The final 1361 bp were obtained as a *Nco*I/*Hin*dIII fragment from P8, and cloned into the *Nco*I/*Hin*dIII site in BSK(III), creating BSK(IV). The assembled *TANKYRASE 2* cDNA was subcloned into the *Not*I/*Xba*I sites of pcDNA 3.1(+) (Invitrogen, Groningen, The Netherlands).

Coupled transcription/translation reactions were performed according to the manufacturer's instructions (Promega).

*Genomic Localization of TANKYRASE 2*—The original *TANKYRASE 2* cDNA clone (L1) subcloned into pGEX-4T-2 (Amersham Pharmacia Biotech) was nick-translated with biotin-14-dATP and hybridized *in situ* at a final concentration of 15 ng/$\mu$l to metaphases from two normal males. The FISH method was modified from that described previously (25) in that chromosomes were stained before

This system allows for the possibility that the same variant will be identified in different species, and should therefore be given the same isoform designation (indicated by a Greek letter).

analysis with both propidium iodide (as counterstain) and DAPI (for chromosome identification). Images of metaphase preparations were captured by a cooled charged coupled device camera using the ChromoScan image collection and enhancement system (Applied Imaging Corporation, Newcastle, United Kingdom). FISH signals and the DAPI banding pattern were merged for figure preparation.

*Northern Blot Analysis*—Human multiple tissue Northern blots (CLONTECH) were hybridized under conditions recommended by the manufacturer. The radiolabeled probe utilized was the *TANKYRASE 2* cDNA clone L1 labeled by random primer extension (Promega) using [$\alpha$-$^{32}$P]dCTP (Amersham Pharmacia Biotech).

*Generation of GST Fusion Proteins*—The following regions of tankyrase 2 were expressed as GST fusion proteins; amino acids 324–980 (corresponding to clone L1 and construct 1 in Fig. 8), amino acids 324–870 (construct 2), amino acids 324–630 (construct 3), amino acids 631–980 (construct 4, also used to generate Ab-1), amino acids 486–630 (used to generate Ab-5), and amino acids 871–935 (construct 5). Construct 1 was generated by subcloning a *SalI-XhoI* fragment from pACT2 into the *NdeI* site of pGEX-4T-2 (Amersham Pharmacia Biotech). DNA fragments encoding the other regions were synthesized by polymerase chain reaction using flanking primers containing restriction enzyme sites for in-frame directional insertion into this vector. Following cloning and transformation of the resulting plasmids into *Escherichia coli* DH5$\alpha$, GST fusion proteins were purified from isopropyl-$\beta$-D-thiogalactopyranoside-induced bacterial cultures as described previously (26).

*Cell Culture*—DU145 human prostate cancer cells, HEK293 cells, and HEK293 cells stably transfected with the *GRB14*/pRcCMV$_F$ expression vector were maintained as described previously (21). Where indicated, the cells were starved overnight in medium containing 0.5% fetal calf serum.

*Cell Lysis, Immunoprecipitation, and Western Blotting*—These techniques were as described previously (27), except that the lysis and wash buffers used for detection of Grb14-tankyrase 2 co-immunoprecipitation contained 0.1% Triton X-100.

*Cell Fractionation*—DU145 cells were serum-starved overnight in RPMI/0.5% fetal calf serum and then harvested (1 ml/150-mm dish) in subcellular fractionation buffer (250 mM sucrose, 10 mM Tris, pH 7.5, 0.5 mM EDTA, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The cell suspensions were subjected to three freeze-thaw cycles and then Dounce homogenization until, by microscopic inspection, the majority of the nuclei were released. The samples were then centrifuged at 800 × *g* for 10 min (to isolate the low speed pellet), 50,000 × *g* for 20 min (to isolate the HDM), and 160,000 × *g* for 70 min (to isolate the LDM). The pellets from each centrifugation step were resuspended in subcellular fractionation buffer at 10% of the original volume, and the remaining supernatant was then concentrated to the same volume using a Microcon YM-10 centrifugal filter device (Millipore Corp., Bedford, MA). Equivalent amounts of each fraction (*i.e.* normalized for cell number) were then analyzed by Western blotting.

*Affinity Purification of Tankyrase 2 Antisera*—GST or the appropriate GST fusion protein were purified on glutathione-agarose beads (Sigma, Castle Hill, New South Wales, Australia) (26) and then cross-linked to the beads using dimethylpimelimidate (Sigma) (28) to generate affinity columns. The rabbit antiserum was diluted 1:10 with 10 mM Tris-HCl, pH 7.5, and applied to the GST column. The flow-through was then applied to the GST fusion protein column. Following washing with 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, the bound antibodies were eluted with 100 mM glycine, pH 2.5, and immediately neutralized with 1 M Tris-HCl, pH 8.0. The antibodies were finally subjected to buffer exchange with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl using a Centricon 30 microconcentrator (Amicon, Beverly, MA) and stored in aliquots at −70 °C.

*Commercial Antibodies*—Commercially available antibodies used were as follows: M2 monoclonal anti-FLAG antibody (Sigma), monoclonal anti-golgi 58 kDa protein FTCD (29) (Sigma), monoclonal anti-GAL4 AD antibody (CLONTECH), monoclonal anti-GAL4 DNA-BD antibody (CLONTECH), goat polyclonal anti-Grb14 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and D8 polyclonal anti-Flag antibody (Santa Cruz Biotechnology).

*Binding Assays Using GST Fusion Proteins*—Five $\mu$g of GST or GST fusion protein immobilized on glutathione-agarose beads were incubated with 400 $\mu$l of lysate (~5 mg/ml total protein) from serum-starved HEK/Grb14 cells (21) for 2 h at 4 C. The beads were then washed three times with cell lysis buffer and subjected to SDS-PAGE. Following transfer to a polyvinylidene difluoride membrane, bound Grb14 was detected by Western blotting with antibody D8 against the Flag epitope tag.

*Indirect Immunofluorescence*—Cells grown on culture slides in RPMI/10% fetal calf serum were rinsed twice in PBS, fixed at room temperature in 3.7% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.2% Triton X-100 in PBS for 10 min. After extensive washing, fixed cells were blocked in 10% normal goat serum or 2% bovine serum albumin in PBS containing 0.05% Tween 20 at room temperature for 45 min and subsequently incubated with antibodies against tankyrase 2 (Ab-5, 1:50) and Golgi 58-kDa protein (1:50) for 1 h at room temperature or overnight at 4 C. After extensive washes in PBS containing 0.05% Tween 20, cells were incubated with Alexa Fluor® 594-conjugated goat anti-rabbit IgG antibody (1:50, Molecular Probes Inc, Eugene, OR) and Alexa Fluor® 488-conjugated goat anti-mouse IgG antibody (1:50, Molecular Probes) for 45 min at room temperature. To detect Grb14, cells were stained as above with anti-Grb14 antibody (1:100), followed by Texas Red-conjugated donkey anti-goat antibody (1:50, Jackson Immunoresearch Laboratories Inc, West Grove, PA). Following washing, samples were mounted in Vectashield plus DAPI (Vector Laboratories Inc., Burlingame, CA). Images were acquired on a Leica DMR microscope (Leica Microsystems Pty Ltd, Gladesville, New South Wales, Australia) using the TCS SP software.

## RESULTS

*Identification of Grb14-interacting Proteins by the Yeast Two-hybrid Technique*—In order to identify binding partners for the Grb14 adaptor protein, a human liver cDNA library in the Gal4 AD vector pACT2 was screened using a full-length Grb14 bait expressed as a Gal4 DNA-BD fusion. From a screen of ~1 × $10^6$ clones, 31 colonies were initially selected on synthetic complete −Leu−His−Trp medium and were then tested for $\beta$-galactosidase activity. Nine clones gave significant activity in the latter assay and were characterized by DNA sequencing. One of these pACT2 clones harbored a novel cDNA of 1971 bp. This clone encoded a polypeptide of 657 amino acids in frame with the Gal4 DNA-BD and exhibited homology to tankyrase (19), but the absence of translation start and stop codons revealed that the cDNA clone was incomplete.

*Cloning and Characterization of TANKYRASE 2*—Screening of cDNA libraries using the original *TANKYRASE 2* clone L1 as probe led to the isolation of a series of overlapping cDNA clones, which provided the full-length *TANKYRASE 2* cDNA sequence.[3] This revealed an open reading frame for tankyrase 2 spanning 1166 amino acids, encoding a polypeptide with a predicted molecular mass of 130 kDa. The protein sequence for tankyrase 2 aligned with that of tankyrase (19) is shown in Fig. 1. The original cDNA clone isolated by the two hybrid screen, clone L1, spans amino acids 324–980 of the full-length sequence.

The major difference between the two proteins is the absence of a HPS domain in tankyrase 2. The molecular architecture of tankyrase 2, starting at the ankyrin repeat region, is then similar to tankyrase. Both proteins possess 24 ankyrin-type repeats, aligned in Smith *et al.* (19), with an overall sequence identity of 83% and sequence similarity of 90%. The major differences between the two proteins in this region occur at the C termini of ankyrin repeats 1, 14, and 24 and the N terminus of repeat 24, where there are five or more non-conservative changes, and the C terminus of repeat 23, where there is a non-conservative change and then an insertion of 7 amino acids in tankyrase 2 relative to tankyrase (Fig. 1). The ankyrin repeat region is then followed by a SAM domain, exhibiting 77% sequence identity and 89% similarity. The most closely related region is the C-terminal PARP homology domain, with 93% sequence identity and 96% similarity. Critical residues required for $NAD^+$ binding and catalysis are entirely conserved.

[3] The nucleotide sequence for the human *TANKYRASE 2* cDNA has been deposited in the GenBank® database under GenBank accession no. AF329696. We note close matches with sequences deposited under the following accession numbers: AF264912, AX029397, AF305081, and AK023746.

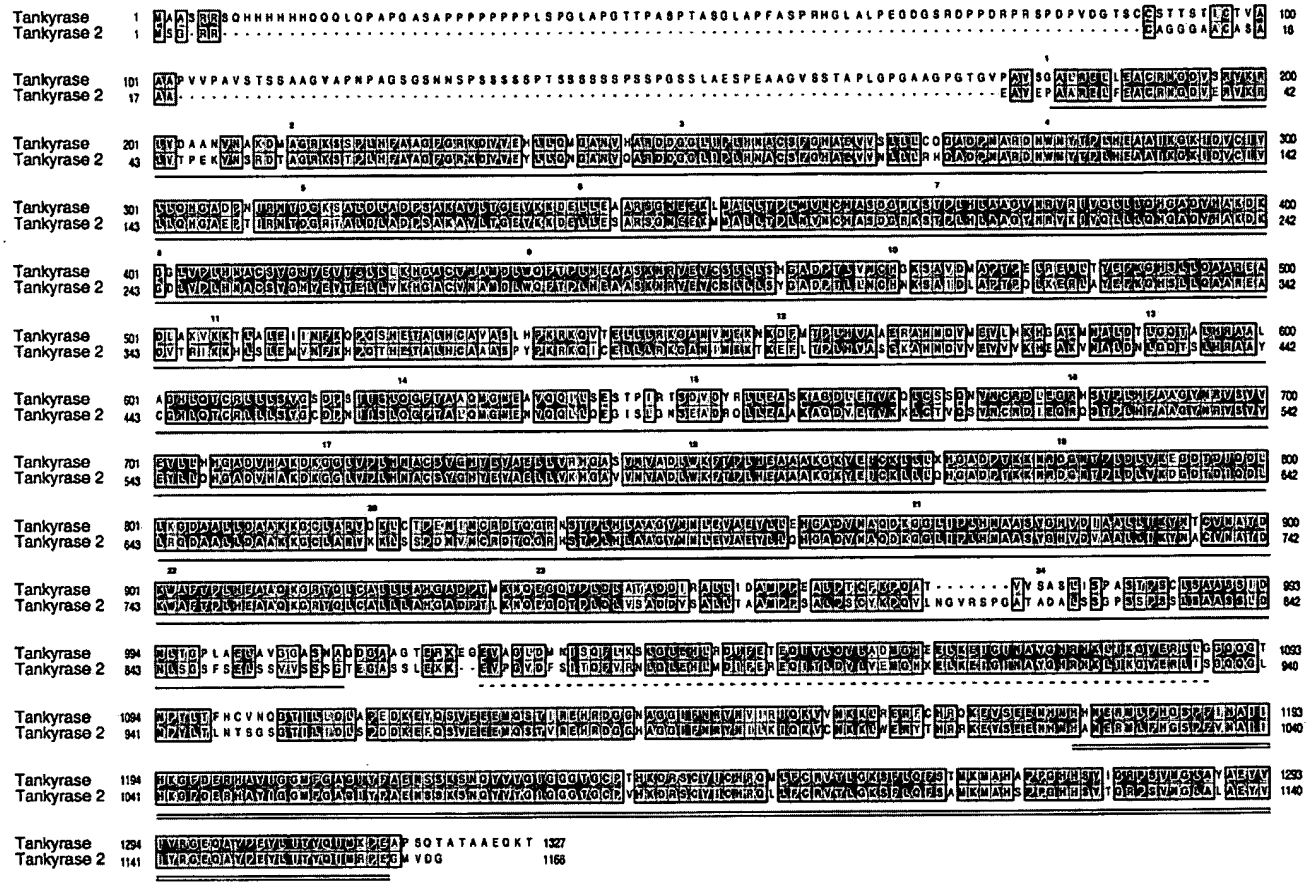



FIG. 1. **Sequence of tankyrase 2 and alignment with tankyrase.** An alignment of the tankyrase and tankyrase 2 protein sequences was generated using the program ClustalW. Identical amino acids (*bold letters, dark shading*) or conservative changes (*light shading*) are *boxed*. The ankyrin repeat region is indicated by a *single underline*, the SAM module by a *broken underline*, and the PARP-related domain by a *double underline*. Note the absence of the N-terminal HPS domain in tankyrase 2. *Numbers above* the ankyrin repeat region indicate the start of individual repeats. Amino acid residues for each protein are *numbered* (from the initiation methionine) on the *left* and *right* of the figure.

*Genomic Localization of TANKYRASE 2*—The *TANKYRASE 2* gene was localized by FISH. Twenty metaphases from a normal male were examined for fluorescent signal. All of these metaphases showed signal on one or both chromatids of chromosome 10 in the region 10q22 to 10q24; 92% of this signal was at 10q23.2 (Fig. 2). There was a total of 13 nonspecific background dots observed in these 20 metaphases. A similar result was obtained from hybridization of the probe to 15 metaphases from a second normal male (data not shown). To increase the mapping resolution, 15 metaphases expressing the folate-sensitive fragile site FRA10A, and showing signal, were then examined. All of these metaphases showed signal proximal to the fragile site. The precise localization of FRA10A was described by Sutherland and Hecht (30) as toward the distal margin of band 10q23.3. Since the *TANKYRASE 2* probe hybridized to band 10q23 and proximal to FRA10A, its likely location is 10q23.2.

*Northern Blot Analysis of TANKYRASE 2 Gene Expression*—The tissue specificity of *TANKYRASE 2* expression was investigated by hybridizing Northern blots of poly(A)$^+$ RNA isolated from a variety of human tissues to a *TANKYRASE 2*-specific cDNA probe. This resulted in the detection of a widely expressed mRNA transcript of ~7 kb (Fig. 3). Expression of *TANKYRASE 2* mRNA was particularly high in skeletal muscle and placenta and moderate in leukocytes, small intestine, ovary, testis, prostate, thymus, spleen, and pancreas. Prolonged exposure of the autoradiograph indicated that expression was low in colon, liver, lung, brain, and heart and undetectable in kidney.

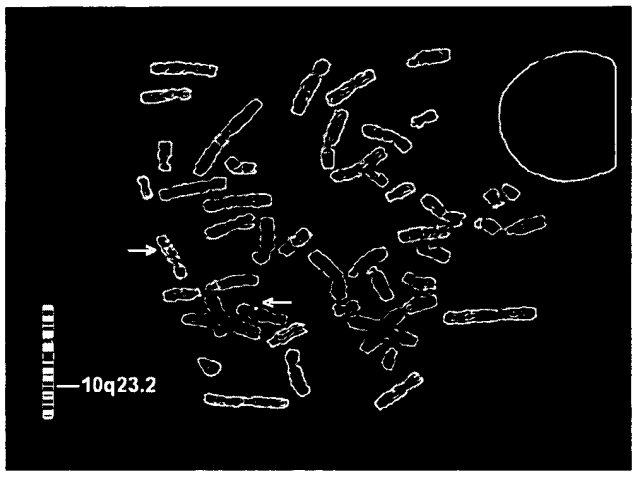



FIG. 2. **Localization of the human *TANKYRASE 2* gene by FISH.** Metaphase showing FISH with the *TANKYRASE 2* probe. The chromosomes have also been stained with DAPI for identification. Hybridization sites on chromosome 10 are indicated by an *arrow*.

*Detection of Tankyrase 2 Protein*—In order to further characterize tankyrase 2, rabbit polyclonal antisera were raised against GST fusion proteins of two non-overlapping regions of the protein. Following affinity purification, these antisera, designated Ab-1 and Ab-5, were used to Western blot cell lysates

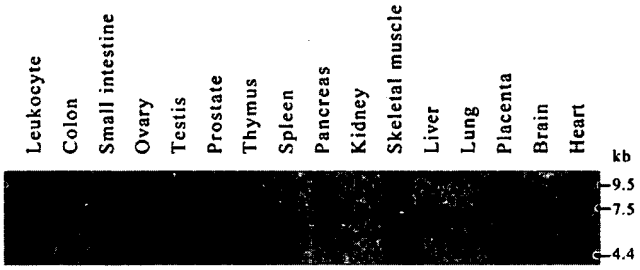



FIG. 3. **Northern blot analysis of *TANKYRASE 2* gene expression.** Northern blots of poly(A)$^+$ RNA isolated from a variety of human tissues were hybridized to a *TANKYRASE 2* cDNA probe labeled with $^{32}$P by random primer extension. The exposure time for the autoradiograph was 16 h with two intensifying screens.

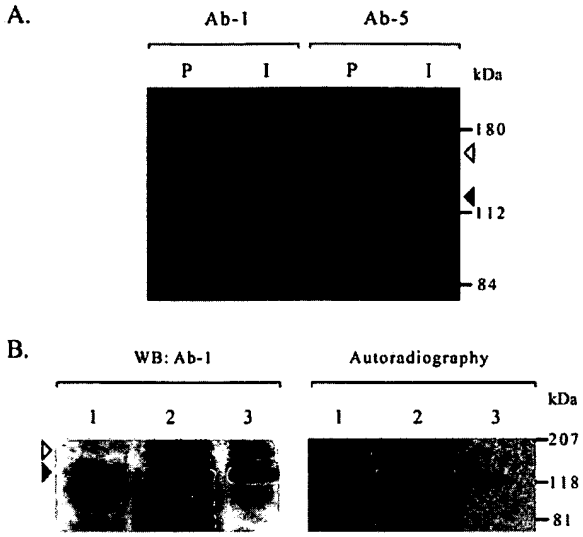



FIG. 4. **Detection of tankyrase 2 protein.** *A*, detection of tankyrase 2 by Western blot analysis. Lysates from DU145 prostate carcinoma cells were separated by SDS-PAGE (6% gel), transferred to nitrocellulose, and Western blotted with either preimmune serum (*P*) or affinity-purified antisera (*I*) Ab-1 or Ab-5. Detection of bound antibody was by ECL. The positions of tankyrase and tankyrase 2 are indicated by *open* and *closed arrowheads*, respectively. *B*, comparison of endogenous tankyrase 2 with that obtained from translation of the *TANKYRASE 2* cDNA. *Left panel*, the following samples were subjected to SDS-PAGE and transferred to nitrocellulose. *Lane 1*, $^{35}$S-labeled transcription-translation reaction programmed with *TANKYRASE 2* cDNA; *lane 2*, Ab-1 immunoprecipitate of sample in *lane 1*; *lane 3*, DU145 cell lysate. The filter was then Western-blotted (*WB*) with Ab-1. Detection of bound antibody was by ECL. *Open* and *closed arrowheads*, as for *A*. The filter was then subjected to autoradiography (*right panel*, 3-day exposure).

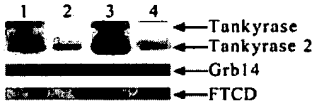



FIG. 5. **Analysis of tankyrase 2 and Grb14 localization by subcellular fractionation.** Low speed pellet (*lane 1*), HDM (*lane 2*), LDM (*lane 3*), and supernatant (*lane 4*) fractions were prepared from DU145 cells as described under "Experimental Procedures." Following separation by SDS-PAGE and transfer to a polyvinylidene difluoride membrane, replicate filters were blotted for tankyrase 2 (using Ab-1) (*top panel*), Grb14 (*middle panel*), and the Golgi 58-kDa marker FTCD (*bottom panel*). Detection of bound antibodies was by ECL.

from DU145 prostate carcinoma cells. This cell line was initially chosen as a model because it expresses high levels of Grb14 (21). Both antisera, and not their respective pre-immune controls, detected a protein of ~130 kDa (Fig. 4*A*), which agrees with the size predicted from the open reading frame of the *TANKYRASE 2* cDNA. The weaker band of ~170 kDa detected on these blots is likely to represent tankyrase, which exhibits close similarity to tankyrase 2 in the regions used for antibody production but, due to the presence of the HPS domain, migrates more slowly upon SDS-PAGE.

In order to compare the size of tankyrase 2 in cells with that translated from the cDNA, a coupled transcription-translation reaction was performed using the full-length *TANKYRASE 2* cDNA in pcDNA 3.1 as template. This reaction produced a specific [$^{35}$S]methionine-labeled product of ~130 kDa, which was immunoprecipitated with Ab-1 (Fig. 4*B*). Furthermore, Western blotting of these samples run adjacent to DU145 cell lysate indicated that this translation product exhibited the same mobility as DU145-derived tankyrase 2, confirming that the open reading frame encoded the full-length protein. The lower molecular mass bands in *lanes 1* and *2* of *both panels* are probably due to the use of downstream translational start sites as the sequence surrounding the initiation methionine is very GC-rich.

*Subcellular Localization of Tankyrase 2*—Although a small fraction of the cellular tankyrase pool localizes to telomeres (19), the majority appears to reside in the cytoplasm in a perinuclear location (20, 31). In 3T3-L1 fibroblasts, tankyrase co-localizes with the Golgi marker FTCD by immunofluorescence and co-fractionates with this marker upon subcellular fractionation (20). We were therefore interested in determining the subcellular localization of tankyrase 2. DU145 cells were separated by centrifugation into low speed pellet (residual intact cells, nuclei), HDM (mitochondria, Golgi, endoplasmic reticulum), LDM (vesicles endosomes), and supernatant (cytosol) fractions, which were then analyzed by Western blotting (Fig. 5). Blotting with Ab-1 revealed that both tankyrase (170 kDa) and tankyrase 2 (130 kDa) were predominantly recovered in the LDM fraction, with lower amounts in the HDM fraction and the supernatant. FTCD was mainly recovered in the LDM fraction, with lower amounts in the cytosol, as would be expected for a peripheral membrane protein in the Golgi. Interestingly, Grb14 exhibited a similar distribution to tankyrase 2, preferentially associating with the LDM fraction. These results indicate that tankyrase, tankyrase 2, and Grb14 co-reside in a subcellular fraction enriched in Golgi vesicles and endosomes.

In order to characterize the subcellular location of the tankyrases further, DU145 cells were stained with Ab-5, an antibody against the Golgi marker FTCD, or the two in combination, and then analyzed by confocal microscopy (Fig. 6). The Golgi marker exhibited a perinuclear location, as would be expected for this organelle (Fig. 6*A*) whereas staining with Ab-5 revealed both diffuse and punctate cytoplasmic staining (Fig. 6*B*). This staining was more widespread than the FTCD staining, often extended to more peripheral regions of the cell, and generally did not co-localize with FTCD upon overlay (Fig. 6*C*). Similar results were obtained using Ab-1. These results therefore differ from those presented by Chi and Lodish (20), where tankyrase strongly co-localized with the Golgi marker FTCD. This may reflect a cell type difference (Chi and Lodish used 3T3-L1 fibroblasts) or preferential detection of tankyrase 2 by our antibody upon immunostaining, with tankyrase 2 exhibiting a different distribution to tankyrase. Since, in DU145 cells, both tankyrases are enriched in the LDM fraction (Fig. 5), the punctate staining obtained in Fig. 6*B* may represent early endosomes.

Unfortunately, we were unable to establish conditions for co-staining of DU145 cells for both tankyrase 2 and Grb14. However, indirect immunofluorescence using an anti-Grb14 antibody revealed largely diffuse, but also punctate, cytoplasmic staining that was more concentrated around the nucleus (Fig. 6*D*). A pool of Grb14 also localized to the plasma membrane. Although plasma membrane localization was not ob-

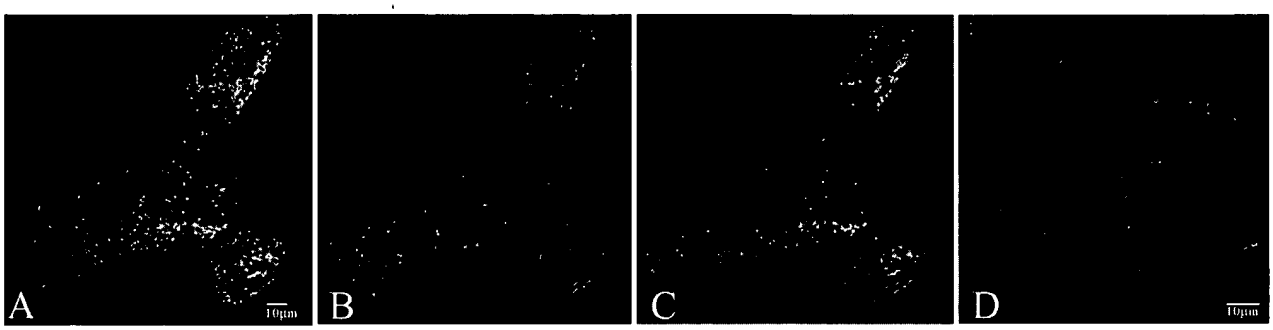


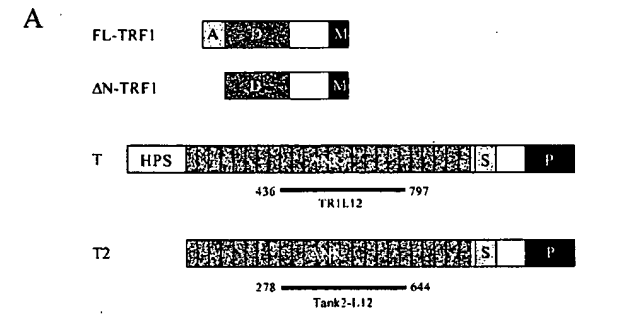

FIG. 6. **Immunolocalization of tankyrases and Grb14 in DU145 prostate carcinoma cells.** Cells were fixed in 3.7% paraformaldehyde and stained with antibodies raised against the Golgi 58-kDa protein FTCD (*A*; green Alexa Fluor 488) and tankyrase 2 (*B*; red Alexa Fluor 594) as described under "Experimental Procedures." The overlay is shown in *C*, and any co-localization between tankyrases and FTCD appears *yellow*. In *panel D*, cells were stained with an anti-Grb14 antibody followed by a Texas Red-conjugated secondary antibody.

served for the tankyrases, the staining pattern for the tankyrases and Grb14 is consistent with the interaction of these proteins in the cytoplasm.

*Comparison of the Binding Selectivity of the Ankyrin Repeat Regions of Tankyrase 2 and Tankyrase*—The interaction of tankyrase 2 with Grb14, coupled with its predominantly cytoplasmic localization, led us to investigate whether the properties of this protein might differ from tankyrase, which binds the telomeric protein TRF1 (19). A region of tankyrase containing 10 consecutive ankyrin repeats (denoted TR1L12) is sufficient for binding the N-terminal acidic domain of TRF1 (19). In order to investigate whether the corresponding region of tankyrase 2 binds TRF1, this fragment (denoted Tank2-L12 and encompassing amino acids 278–644; Fig. 7*A*) was expressed as a Gal4 AD fusion in yeast together with a LexA-TRF1 bait. Interestingly, this region of tankyrase 2 did not bind TRF1, whereas a strong induction of β-galactosidase activity occurred with positive controls involving either TR1L12-TRF1 interaction or TRF1 dimerization (Fig. 7*B*). Western blotting of yeast extracts confirmed expression of the appropriate fusion proteins. Consequently, although tankyrase and tankyrase 2 are closely related in their ankyrin repeat regions (90% amino acid similarity), the small number of amino acid substitutions present are sufficient to markedly alter the binding selectivity of these protein-protein interaction domains.

*A Subset of Tankyrase 2 Ankyrin Repeats Is Sufficient for Binding Grb14*—The original tankyrase 2 cDNA clone isolated by the two hybrid screen (clone L1, corresponding to construct 1 in Fig. 8*A*) stretched from midway through ankyrin repeat 10 to the region between the SAM and PARP domains. This region also bound Grb14 in a GST fusion protein pull-down assay (Fig. 8*B*). In order to further delineate a Grb14 binding region, a series of deletion constructs were generated and used in this assay (Fig. 8). We accept that regions of tankyrase 2 outside of construct 1 may also contribute to binding of full-length tankyrase 2 to Grb14. Deletion of the SAM domain (construct 2) did not affect Grb14 binding, and the SAM domain alone (construct 5) did not bind Grb14, indicating that the SAM domain is dispensable for Grb14 interaction. However, construct 4, encoding the C terminus of repeat 19 through to, and including, the SAM domain did not bind Grb14, implicating the N-terminal set of repeats in Grb14 binding. This was confirmed using a GST fusion protein corresponding to a region between mid-repeat 10 and mid-repeat 19 (construct 3), which bound Grb14 almost as strongly as construct 1.

*The N Terminus of Grb14 Is Sufficient for Tankyrase 2 Binding*—In order to map the regions of Grb14 involved in binding to tankyrase 2, a series of Grb14 deletion mutants fused to the Gal4 DNA-BD (Fig. 9) were co-expressed in the yeast strain Y190 with the Gal4 AD fusion of tankyrase 2 isolated by the




B

| LexA-fusion | AD-fusion: V | TR1L12 | Tank2-L12 | FL-TRF1 |
|---|---|---|---|---|
| FL-TRF1 | 5.2 | 2.76 x $10^3$ | 7.5 | 5.99 x $10^3$ |
| ΔN-TRF1 | 5.0 | 4.6 | 4.8 | ND |

FIG. 7. **Comparison of the binding selectivity of the tankyrase and tankyrase 2 ankyrin-repeat regions.** *A*, schematic representation of the TRF1, tankyrase, and tankyrase 2 fusion partners utilized in yeast two hybrid analysis. In TRF1, *A* denotes the acidic region, *D* the dimerization domain, and *M* the Myb-related region. Full-length TRF1 (FL-TRF1) or the N-terminal truncation ΔN-TRF1 were fused to LexA. In tankyrase (*T*) and tankyrase 2 (*T2*), *ANK* denotes the ankyrin repeat region, *S* the SAM module, and *P* the PARP homology domain. The *solid bars* represent the 10-ankyrin repeat region of tankyrase responsible for TRF1 binding (TR1L12) and the corresponding region of tankyrase 2 (Tank2-L12) with the amino acid boundaries indicated. These two regions were fused to the Gal4 AD. *B*, analysis of the interaction between the ankyrin repeat regions of tankyrase or tankyrase 2 with TRF1. TR1L12 and Tank2-L12 were expressed as Gal4 AD fusions in yeast strain L40 with the TRF1 or ΔN-TRF1 bait constructs. The AD vector pGAD10 (*V*) was used as a negative control. Interaction of LexA and the Gal4 AD via TRF1 dimerization was used as an additional positive control. β-Galactosidase activity assays were performed by the liquid culture-derived method and represent the mean of triplicate determinations. Results are expressed as mean β-galactosidase units ($\times 10^{-3}$). *ND*, not determined.

two hybrid screen. Deletion of the SH2 domain did not markedly affect binding of tankyrase 2 (Fig. 9). In contrast, removal of the N-terminal region prevented tankyrase 2 interaction. Furthermore, expression of a fusion protein containing only the Grb14 N terminus resulted in significant β-galactosidase activity, demonstrating that this region is not only required, but also sufficient, for tankyrase 2 binding. However, since we did not utilize a construct lacking the N terminus but containing the SH2 domain, we cannot completely rule out a contribution of the SH2 region to Grb14-tankyrase 2 interaction.

*Association of Grb14 with Tankyrase 2 in Vivo*—In order to

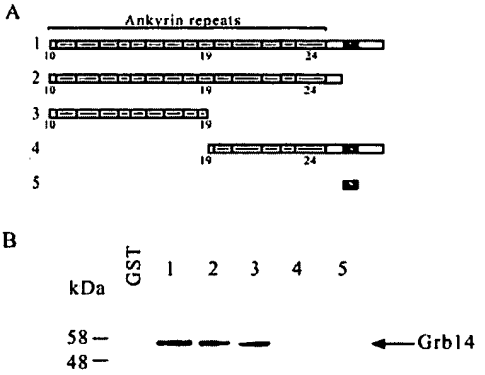



FIG. 8. **Delineation of a Grb14 binding region on tankyrase 2.** *A*, schematic representation of the different regions of tankyrase 2 expressed as GST fusion proteins. The different constructs are *numbered* on the *left* of the figure. The individual ankyrin repeats are drawn to scale, and key repeats are labeled. The region labeled *S* is the SAM domain. *B*, mapping of a Grb14-binding region on tankyrase 2 by a GST fusion protein pull-down assay. The fusion proteins illustrated in *A* were coupled to glutathione-agarose beads and incubated with lysates from cells expressing Flag epitope-tagged Grb14. Following washing of the beads, bound Grb14 was detected by Western blotting with an antibody against the epitope tag.

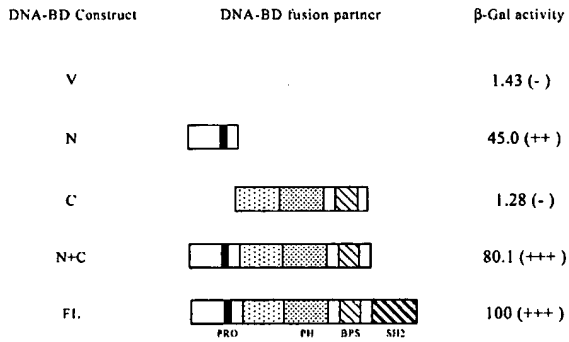



FIG. 9. **Mapping of the tankyrase 2 binding site on Grb14.** Constructs encoding fusions of full-length Grb14 (*FL*), the N-terminal region (*N*), central region (*C*), and N-terminal + central regions (*N+C*) were generated in the vector pAS2–1. The table shows results of β-galactosidase activity assays following transformation of plasmids encoding the above Grb14-BD fusions or pAS2–1 vector alone (*V*) into yeast strain Y190 together with a tankyrase 2 cDNA clone in pACT2 encoding amino acids 324–980 (clone L1). Assays were performed in triplicate by the liquid culture-derived method and expressed as a percentage relative to the activity obtained with the full-length Grb14 construct. Additionally, the results of a colony lift filter assay are shown in *parentheses*, with the intensity of blue color development scored from – (absent) to +++ (strong).

investigate whether Grb14 associates with tankyrase 2 in living cells, HEK-293 cells stably transfected with Flag epitope-tagged Grb14 (21), were utilized. These cells express tankyrase 2 but at ~5-fold lower levels than DU145 cells, and subcellular fractionation of these cells revealed that, as in DU145 cells, both tankyrase 2 and Grb14 preferentially associate with the LDM fraction.[4] Anti-Flag immunoprecipitations were performed on lysates from cells transfected with Grb14 or control HEK293 cells, and the immunoprecipitates then Western-blotted for the presence of tankyrase 2 (Fig. 10). This led to the detection of tankyrase 2 in the immunoprecipitate containing Grb14, but not in the control immunoprecipitate. On longer exposure of the autoradiograph, tankyrase was also detected in the Grb14 immunoprecipitate.[4] However, at this stage, we do not know whether the Grb14-tankyrase association is direct.

[4] R. J. Lyons, R. Deane, G. M. Sanderson, and R. J. Daly, unpublished results.

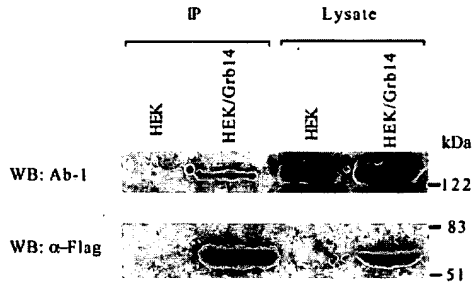



FIG. 10. **Tankyrase 2 and Grb14 associate *in vivo*.** Lysates from serum-starved control HEK293 cells (*HEK*) or HEK293 cells stably transfected with Flag epitope-tagged Grb14 (*HEK/Grb14*) were subjected to immunoprecipitation with the anti-Flag monoclonal antibody M2. The immunoprecipitates (*IP*) were then separated by SDS-PAGE, transferred to nitrocellulose, and Western-blotted (*WB*) with anti-Flag antibodies (M2) or anti-tankyrase 2 antibodies (Ab-1). Lysates from these cell lines were analyzed in parallel.

This association of Grb14 with tankyrase 2 *in vivo* suggests that the interaction of these two proteins detected in GST pull-down assays (Fig. 8) and in the yeast two-hybrid system (Fig. 9) is physiologically relevant.

## DISCUSSION

Both the signaling mechanism and function of the Grb7 family of adaptor proteins are currently poorly understood, but the identification of non-receptor tyrosine kinase binding partners for their conserved protein modules may shed light on both these properties. Such interacting molecules may perform effector roles or regulate such processes, for example, by modulating a catalytic activity or regulating subcellular localization. To date, two studies have identified candidate proteins for such roles. The serine/threonine kinases Raf1 and MEK1 (32) and the E3 ubiquitin ligase Nedd4 (33) associate with Grb10, these interactions being mediated primarily by the Grb10 SH2 domain in a phosphotyrosine-independent manner. However, tankyrase 2 represents the first protein identified that binds the N terminus of a Grb7 family protein. Deletion analysis identified the N-terminal 110 amino acids of Grb14 as being sufficient for this interaction (Fig. 9). The N-terminal region contains the conserved proline-rich motif of the Grb7 family (PSIPNPFPEL in Grb14) flanked by two short stretches of charged amino acids (21). We have yet to determine the relative contribution of these two types of sequence motif to tankyrase 2 binding, although it is interesting that the binding partner identified for this region of Grb14 does not contain an SH3 or WW domain, protein-protein interaction modules that target specific proline-rich sequences (34). In GST pull-down experiments, a region of tankyrase 2 encompassing amino acids 324–630 (ankyrin repeats 10–19) was sufficient for binding Grb14 (Fig. 8). These experiments also demonstrated that the SAM domain of tankyrase 2 is not involved in Grb14 interaction. The role of the SAM domain in the tankyrases is not known, but these domains can mediate both homotypic and heterotypic protein-protein interactions (35).

Our identification of a second ankyrin repeat-containing PARP is interesting in the context of the possible evolutionary relationship between the genes encoding the tankyrases and ankyrins. The localization of both the *TANKYRASE* (*TNKS*) and *ANK1* genes to chromosome 8 and the significant structural and sequence homology of the repeat regions of the two encoded proteins led Zhu *et al.* (36) to suggest a common ancestral origin for these two genes. The localization of the *TANKYRASE 2* gene to chromosome 10q23.2, close to the *ANK3* gene at 10q21 (37), provides further support for this hypothesis and indicates that these two gene families have

co-segregated during evolution. Both tankyrase-encoding genes are widely expressed, but *TANKYRASE* expression, unlike that of *TANKYRASE 2* (Fig. 3) is very high in testis compared with other tissues (19), arguing for some functional specificity. The major structural difference between tankyrase and tankyrase 2 is the absence of a HPS domain in the latter (Fig. 1). The remainder of the proteins possess the same domain structure and are highly related in amino acid sequence (91% amino acid similarity). The role of the HPS domain is not known at present, although the identification of tankyrase as a substrate for growth factor-induced MAP kinase activity (20) and the presence of four PXSP consensus sites for phosphorylation by MAP kinases (38) in the HPS domain, suggest that this region may be targeted by these enzymes. It is interesting that the ankyrin repeat regions are highly related but exhibit a different binding selectivity, in that a 10-ankyrin repeat segment of tankyrase, but not tankyrase 2, binds TRF1 in a two hybrid assay (Fig. 7). The region involved spans repeats 9–18, and sequence comparisons suggest that a divergent stretch of amino acids at the C-terminal end of repeat 14 may be responsible for this difference in binding activity (Fig. 1). We have yet to determine whether the ankyrin repeat region of tankyrase binds Grb14. However, it is noteworthy that the TRF1-binding region of tankyrase (ankyrin repeats 9–18) is very similar to a Grb14-binding region of tankyrase 2 (ankyrin repeats 10–19, Fig. 8), suggesting that this region of the tankyrases presents a binding surface for protein-protein interaction.

Smith and de Lange (31) localized tankyrase in HeLa cells to telomeres, nuclear pore complexes, or the pericentriolar matrix, depending on the stage of the cell cycle. However, under certain staining conditions, a strong punctate juxtanuclear staining was also evident. The recent work of Chi and Lodish (20) indicates that this is probably due to a pool of tankyrase in the Golgi, since tankyrase in 3T3-L1 fibroblasts associated with the LDM fraction upon subcellular fractionation and co-localized with the Golgi marker FTCD by immunofluorescence. Furthermore, tankyrase bound the cytoplasmic domain of IRAP *in vitro*, and in 3T3-L1 adipocytes co-localized with perinuclear, but not cytoplasmic, Glut4. Unfortunately, we have not been able to raise a tankyrase 2-specific antibody to allow definitive determination of the subcellular location of this protein by immunofluorescence, as an anti-peptide antibody against amino acids 811–830, which are divergent between tankyrase and tankyrase 2 (Fig. 1), exhibited very low affinity binding.[4] However, our subcellular fractionation studies indicate that, in DU145 cells, both tankyrase and tankyrase 2 associate with the LDM fraction (Fig. 5), and indirect immunofluorescence using an antibody that recognizes both tankyrase species detects punctate cytoplasmic staining, which generally does not co-localize with the Golgi marker FTCD (Fig. 6). The difference between our results and those of Chi and Lodish may reflect cell type specificity in localization and/or differences in the localization of tankyrase and tankyrase 2. However, it is now clear that the function of the tankyrases is not restricted to the nucleus, and that they must also perform a function in the cytoplasm where they associate with the Golgi or endosome fractions. The lack of association of the ankyrin repeat region of tankyrase 2 with the telomeric protein TRF1 (Fig. 7) may indicate that tankyrase 2 does not function at telomeres, but further experimentation will be required to resolve this issue.

What may be the function of the tankyrases in the cytoplasm? Tankyrase exhibits PARP activity *in vitro* toward both itself and the exogenous substrates TRF1 and the IRAP cytoplasmic domain (19, 20), although endogenous substrates in cells have yet to be identified. The high conservation of tankyrase 2 in the PARP homology domain (Fig. 1), including the essential residues for catalytic activity (19), strongly suggests that tankyrase 2 will also possess PARP activity. Interestingly, the activity of C-terminal-binding protein 1/brefeldin A-ADP-ribosylated substrate, which acylates lysophosphatidic acid to promote the fission of Golgi membranes, is inhibited by ADP-ribosylation (39), and this protein becomes ADP-ribosylated by an unknown activity upon treatment of cells by the Golgi-disrupting agent brefeldin A (40). Consequently, ADP-ribosylation may regulate vesicle formation in this and other cytoplasmic compartments. As indicated by the mode of tankyrase 2 binding to Grb14, the repeat region of the tankyrases is likely to participate in protein-protein interactions in a manner similar to that of the ankyrins themselves, which link specific integral membrane proteins to the underlying cytoskeleton (41). A precedent for the interaction of ankyrins with signaling proteins associated with the plasma membrane is the binding of the ankyrin-repeat regions of Ank1 and Ank3 to the Rho family GDP-GTP exchange factor Tiam 1 (42). However, the ankyrins are not restricted to a plasma membrane location. Particular ankyrin isoforms, some retaining the ankyrin-repeat region, are associated with the Golgi and endolysosomes, where they form part of a Golgi-associated spectrin skeleton, which may function in structural organization, cargo selection, and/or linkage to motor proteins (43). Finally, an intriguing observation in relation to the potential role of tankyrase 2 in regulating vesicle dynamics is that a region of mouse chromosome 19 syntenic with human chromosome 10q23 (which harbors *TANKYRASE 2*, Fig. 2) contains two loci, *pale ear* (*ep*) and *ruby-eye* (*ru*), responsible for phenotypes similar to human Hermansky-Pudlak syndrome, a condition associated with defects in multiple cytoplasmic organelles, including lysosomes (44, 45). The *ep* locus corresponds to the human gene *HPS*, which is mutated in a subset of Hermansky-Pudlak syndrome patients (45). However, the gene responsible for the defect in *ru* mice remains to be identified, and our studies highlight *Tankyrase 2* as a candidate.

A surprising result was that Grb14 preferentially associates with the LDM fraction (Fig. 5). Since Grb14 associates with tankyrase 2 in GST pull-down assays (Fig. 8), the yeast two hybrid system (Fig. 9) and *in vivo* (Fig. 10), tankyrase 2 may tether Grb14 to vesicles in this fraction, but the PH domain of Grb14 may also participate. What could be the significance of this localization? Conceivably, vesicle trafficking may deliver Grb14 to a specific subcellular location. A precedent for this is that movement of paxillin from the Golgi apparatus to focal adhesions is dependent on the activity of the small GTP-binding protein ARF1, which regulates coatamer assembly on Golgi transport vesicles (46). Alternatively, given the association of Grb14 with specific activated growth factor receptors (12, 13), the tankyrase 2-Grb14 interaction may play a role in sorting of internalized receptors. In this context it is interesting that we also isolated Nedd4, which regulates the ubiquitination and endocytosis of cell surface proteins (47), as a Grb14 binding partner in our two hybrid screen. Similar to the Grb10-Nedd4 interaction reported by Morrione *et al.* (33), the SH2 domain of Grb14 binds *in vitro* to a region of Nedd4 encompassing the calcium-lipid binding/C2 domain in a phosphotyrosine independent manner, although to date we have been unable to co-immunoprecipitate these two proteins.[4] These hypotheses concerning the signaling mechanism of Grb14 are currently under investigation.

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